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Natural Exon Skipping Sets the Stage for Exon Skipping as Therapy for Dystrophic Epidermolysis Bullosa

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Dystrophic epidermolysis bullosa (DEB) is a devastating blistering disease affecting skin and mucous membranes. It is caused by pathogenic variants in the *COL7A1* gene encoding type VII collagen, and can be inherited dominantly or recessively. Recently, promising proof-of-principle has been shown for antisense oligonucleotide (AON)-mediated exon skipping as a therapeutic approach for DEB. However, the precise phenotypic effect to be anticipated from exon skipping, and which patient groups could benefit, is not yet clear. To answer these questions, we studied new clinical and molecular data on seven patients from the Dutch EB registry and reviewed the literature on *COL7A1* exon skipping variants. We found that phenotypes associated with dominant exon skipping cannot be distinguished from phenotypes caused by other dominant DEB variants. Recessive exon skipping phenotypes are generally relatively mild in the spectrum of recessive DEB. Therefore, for dominant DEB, AON-mediated exon skipping is unlikely to ameliorate the phenotype. In contrast, the overall severity of phenotypes associated with recessive natural exon skipping pivots toward the milder end of the spectrum. Consequently, we anticipate AON-mediated exon skipping for recessive DEB caused by bi-allelic null variants should lead to a clinically relevant improvement of this devastating phenotype.

INTRODUCTION

Dystrophic epidermolysis bullosa (DEB) is a monogenic, heritable skin disease caused by pathogenic variants in the *COL7A1* gene, which encodes the epidermal-dermal adhesion protein type VII collagen. DEB, which can be inherited either autosomal dominantly (DDEB; OMIM: 131750) or recessively (RDEB; OMIM: 226600), is characterized by blistering of skin and mucosae upon the slightest trauma.[1] The severity of DEB is generally well correlated to the quantity and functionality of type VII collagen that is expressed.[2] Phenotypic severity ranges from involving only nails (DDEB-na) to generalized and severe blistering and scarring (RDEB-gen sev). In general, the prognosis of DDEB is much better than that of RDEB. Patients suffering from RDEB-gen sev often die at the age of 30–40 years because of complications of blister formation and aggressive squamous cell carcinomas evoked by the complete absence of type VII collagen.[3] Patients suffering from milder DEB phenotypes in general have a normal lifespan. Currently, several therapeutic strategies are under investigation, although no curative therapy has been translated into the clinic and treatment remains primarily symptomatic.[4]

Type VII collagen is an extracellular matrix component that secures the epidermis to the papillary dermis by forming anchoring fibrils. The 118 exons of *COL7A1* are first translated into a pro-α1-type VII collagen molecule, which comprises an ∼145-kDa amino-terminal non-collagenous 1 domain (NC-1), an ∼145-kDa central triple-helix domain (THD), and an ∼30-kDa C-terminal non-collagenous 2 domain (NC-2). The NC-1 domain contains regions that are essential for interactions to binding partners like type IV collagen and laminin-332.[5,6] The THD contains a highly repetitive glycine-Xaa-Yaa amino acid sequence (Gly-X-Y) that is essential for proper triple-helix formation.[7] The THD, encoded by exons 29–112, is interrupted 19 times, with the flexible, so-called intrinsically disordered, central 39-amino acid "hinge region" being the major interruption.[8] Post-translational modifications subsequently lead to triple-helix formation of three pro-α1 chains, followed by cleavage of the NC-2 domain and antiparallel dimerization. Extracellularly, the antiparallel dimers aggregate laterally to form anchoring fibrils.

DEB-causing variants are widely spread throughout the 118 exons of the *COL7A1* gene. In total, more than 700 DEB-causing variants in the *COL7A1* gene have been reported.[9,10] Variants that lead to dominant phenotypes are exclusively located in the THD, where exon 73 is a hotspot.[11] A single glycine substitution in the THD can impair the ability to form stable triple helices and is the main cause of DDEB.[12] RDEB-causing variants, on the other hand, are found throughout the

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entire gene. RDEB-gen sev is caused by bi-allelic null variants, whereas milder RDEB phenotypes are due to at least one allele capable of producing an, albeit imperfect, pro-α1 type VII collagen. The precise phenotype is determined by the combination of both alleles and the dynamic interplay with the individual's genetic background.[13]

Multiple *COL7A1* variants are located in splicing signals and can disturb normal splicing.[9,10] Several lead to an entire exon being spliced out from the pre-mRNA, i.e., the exon is skipped. Of the 118 *COL7A1* exons, 107 are "in-frame," and skipping these exons will still leave the reading frame intact. Because the exons of the *COL7A1* gene are very small (27–201 bp), with an average length of 54 bp (NM_000094.3), exon skipping would exclude only short sequences from the mRNA and lead to a type VII collagen that is predicted to be only slightly shorter.

The small exons, in combination with the highly repetitive sequence of the THD, make *COL7A1* an ideal candidate for antisense oligonucleotide (AON)-mediated exon skipping.[14] AON-mediated exon skipping aims to bypass disease-causing *COL7A1* variants by removing the mutant exon and thereby restoring the expression of a functional type VII collagen. The strong correlation between type VII collagen expression and the clinical phenotype predicts that the slightest increase in type VII collagen deposition at the basement membrane zone (BMZ) should have marked effects on the phenotype.[2] Recently, we have shown encouraging pre-clinical results with AON-mediated exon skipping as a systemic therapeutic approach for RDEB.[15] We also showed that exon 13 and 105-skipped type VII collagen retains functionality comparable with wild-type.[14] The precise benefit of exon skipping therapy, and which patient groups would benefit from it, is, however, not yet clear. We hypothesized that studying patients in which *COL7A1* variants induce natural exon skipping would shed light on the therapeutic potential of AON-mediated exon skipping. We therefore scrutinized the Dutch Epidermolysis Bullosa (EB) registry for natural exon skipping variants and reviewed the literature on this class of variants. The overview of the natural exon skipping variants presented here sets the stage for further work on AON-mediated exon skipping therapy for DEB.

RESULTS

Exon Skipping Variants Found in the Dutch EB Registry

There is a large cohort of DEB patients registered at the Center for Blistering Diseases, University Medical Center Groningen (the Netherlands). In June 2018, there were 176 DEB patients, of which 100 had a dominant and 76 a recessive form of DEB. Of these 176 patients, 26 carried variants located in putative splice sites, of which 7 were confirmed to induce in-frame exon skipping at the RNA level by RT-PCR. Six resulted in skipping of an out-of-frame exon, and 13 resulted in aberrant splicing other than skipping an entire exon. A comparison of the clinical presentation and immunofluorescence (IF) staining between the patients carrying those seven in-frame exon skipping variants showed that the phenotypic severity and type VII collagen expression levels at the BMZ varied markedly (Figure 1). The seven patients and their families are described briefly below.

The father and son of family 1 (EB-072) both presented with generalized blistering upon slight trauma, constituting a typical generalized DDEB (DDEB-gen) phenotype; they carried the heterozygous variant c.6181-6T>G in intron 73. It was predicted that this variant would disrupt normal splicing and consequently induce exon 74 skipping, which was indeed confirmed at the RNA level (Figure S1). Because in-frame skipping of exon 74 has been described as exerting a dominant-negative effect over the wild-type allele and causing DDEB,[16] we concluded this natural skipping of exon 74 would explain their DDEB phenotype and inheritance pattern.

The father and daughter of family 2 (EB-152) presented with a DDEB-gen phenotype caused by a heterozygous deletion of the last two nucleotides of exon 87 (c.6899_6900del). This variant abolishes the intron 87 splice donor site, which results in the deletion of the entire exon 87 (Figure S1). A dominant-negative effect of in-frame exon 87 skipping has been described, and this explains their DDEB phenotype.[17–30]

The index patient in family 3 and her father (EB-156) presented with a typical DDEB-acral (DDEB-ac) phenotype (Figure 1): nail dystrophy and blisters predominantly on hands, knees, and feet. An intronic, 21-bp deletion (c.6832-23_6832-3del) was identified heterozygously in intron 86 and was predicted to result in the loss of the intron 86 splice acceptor site. RT-PCR indeed confirmed in-frame exon 87 skipping in this family, explaining their dominant phenotype (Figure S1).

The index patient in family 4 (EB-339) presented with generalized blistering upon minor trauma (DDEB-gen). The heterozygous variant c.7894-2A>G in intron 107 was found both in the patient and his affected mother, and it was predicted to lead to the loss of the intron 107 splice acceptor site. RNA analysis confirmed in-frame skipping of exon 108 (Figure S1). Because no other variant was found and skipping of exon 108 has been described to cause a dominantly inherited phenotype,[31] this most likely explains their phenotype.

DNA analysis of the index patient in family 5 (EB-052) showed compound heterozygosity for the maternal, synonymous variant c.4011G>A in exon 33 and the paternal c.7769G>A (p.(Gly2590Asp)) variant in exon 104. The maternal variant at the last position of exon 33 caused skipping of the exon, as shown by RT-PCR (Figure S1). The index patient presented with blistering after minor trauma from birth and the RDEB-gen intermed (RDEB-generalized intermediate) phenotype. No skin abnormalities were observed in the parents on clinical examination, indicating a recessive effect of the exon 33 skipping variant.

The index patient in family 6 (EB-363) has two unaffected parents and presented with a recessively inherited DEB phenotype (RDEB-gen intermed), characterized by generalized blistering of skin and mucosa upon the slightest trauma, with scar tissue formation and loss of nails. Variant analysis identified the c.1573C>T

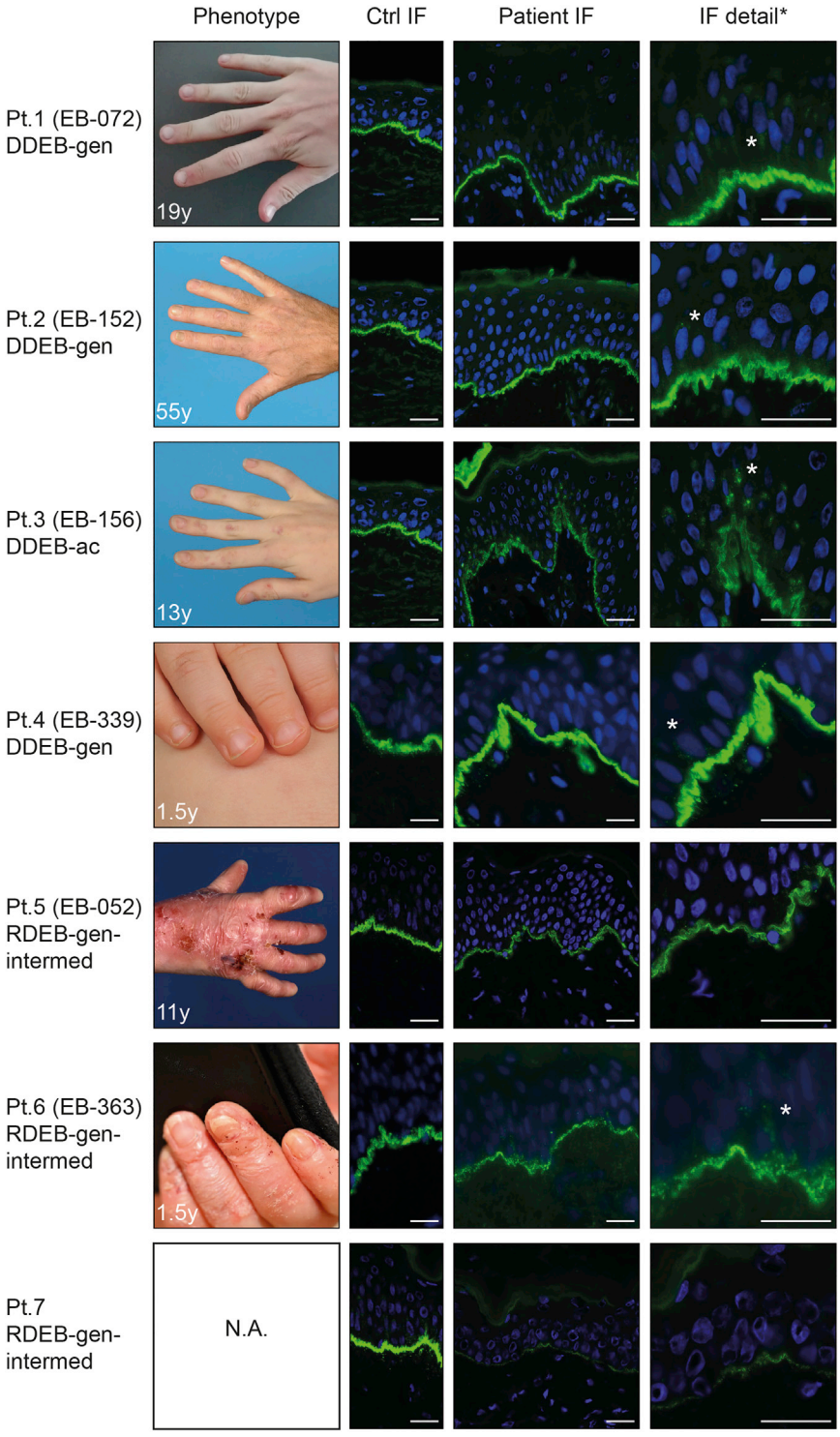

Figure 1. Clinical and Molecular Phenotypes of Seven DEB Patients Associated with Natural Exon Skipping

Left column: DEB clinical phenotypes associated with natural exon skipping depicted by representative photographs of the hands. Immunofluorescence (IF) staining (×40) of type VII collagen at the basement membrane zone (BMZ) is shown compared with control (middle columns). Right column: IF detail of the BMZ revealing retention of type VII collagen by basal keratinocytes (asterisks) in patients 1–4 and 6. Scale bars: 25 μm.

(p.Arg525*) null variant in exon 12 on the maternal allele and the c.8227-1G>C transversion in intron 110 on the paternal allele. RT-PCR revealed that the paternal variant resulted in the in-frame skipping of exon 111 (Figure S1). Because no DEB features could be detected in the father on clinical examination, the in-frame deletion of exon 111 must act recessively, as described previously.[32]

The parents in family 7 were unaffected, but the affected child presented with an RDEB-gen intermed phenotype caused by a homozygous mutation that affected the normal splicing of exon 20. RNA analysis revealed three transcripts: (1) in-frame skipping of exon 20, (2) alternative splicing using a cryptic splice donor in intron 20 resulting in an out-of-frame transcript, and (3) the wild-type transcript (Figure S1). Unfortunately, clinical and molecular details cannot be shown because, despite oral consent being given, we have not received written consent.

Exon Skipping Variants Described in the Literature

Our literature search revealed another 20 *COL7A1* variants that were confirmed at the RNA level to induce in-frame exon skipping, bringing the total to 27 (summarized in Table 1 and Figure 2). This represents 3%–4% of the approximately 700 disease-associated variants that have been described for the *COL7A1* gene. These 27 variants were involved in either dominantly (15/27) or recessively (12/27) inherited phenotypes.

Mechanisms Underlying Exon Skipping

The mechanism underlying exon skipping for all variants is the disruption of constitutional splice signal sequences. The lack of strong, cryptic splice signals in the vicinity of the disrupted constitutive splice signals likely explains why skipping of entire exons occurs instead of other alternative splicing patterns.[33] Out of the 27 natural exon skipping variants, 23 were located in splice

Table 1. Overview of Exon Skipping Variants in DEB

No.	Allele 1[a]	Exon/ Intron	Skipped Exon	Dominant/ Recessive	Allele 2[a]	Exon/ Intron	Skipped Exon	Dominant/ Recessive	Functional Effect on COLVII[b]	Associated Phenotype	IF	References
1	c.[1907G>T;2005C>T] (p.Gly636_Thr683del)	15	15	recessive	c.6311_6312del (p.(Ser2104Trpfs*12)	76	NA	recessive	homogeneous exon 15 skip	RDEB-gen intermed	strongly reduced	35
2	c.2471dup (p.Gly814_Pro863delinsAla)	19	19	recessive	c.2471dup (p.Gly814_ Pro863delinsAla)	19	19	recessive	homogeneous exon 19 skip	RDEB-gen intermed	slightly reduced	20,36,44
				recessive	c.3948dup (p.(Gly1317Trpfs*43))	32	NA	recessive	homogeneous exon 19 skip	RDEB-gen sev	unknown	20,36
3[c]	p.[=, Pro863_Gly904delinsArg])	[c]	20	recessive	p.[=, Pro863_ Gly904delinsArg])	[c]	20	recessive	heterogeneous exon 20 skip	RDEB-gen intermed	slightly reduced	this paper and 5
4	c.4011G>A (p.Gly1326_Pro1337del)	33	33	recessive	c.7769G>A (p.Gly2590Asp)	104	NA	recessive	homogeneous exon 33 skip	RDEB-gen intermed	slightly reduced	this paper (EB-052)
5	c.4980+5G>C (p.Gly1646_Arg1660del)	IVS53	53	recessive	deletion spanning *COL7A1* + 15 other genes	–	NA	recessive	homogeneous exon 53 skip	RDEB-gen intermed	unknown	45
6	c.5820G>A (p.[=, Gly1925_Pro1940del])	70	70	recessive	c.4039G>C (p.Gly1347Arg)	34	NA	recessive	heterogeneous exon 70 skip, mutant	RDEB-ac and RDEB-gen intermed	normal[d]	46,47
				recessive	c.7474C>T (p.(Arg2492*))	98	NA	recessive	heterogeneous exon 70 skip	RDEB-gen intermed	strongly reduced	47
7	c.5856G>C (p.Asn1941_Lys1952del)	71	71	dominant	c. =	NA	NA	NA	heterogeneous exon 71 skip	DDEB-pt	normal	48
8	c.6181-6T>G (p.Gly2061_Gln2072del)	IVS73	74	dominant	c. =	NA	NA	NA	heterogeneous exon 74 skip	DDEB-gen	normal[d]	this paper (EB-072)
9	c.6215del (p.Gly2061_Gln2072del)	74	74	dominant	c. =	NA	NA	NA	heterogeneous exon 74 skip	DDEB-pr	normal	16
10	c.6348+1G>A (p.Val2094_Lys2116del)	IVS76	76	recessive	c.5797C>T (p.Arg1933*)	NA	NA	recessive	homogeneous exon 76 skip	RDEB-pr	normal	49
11	c.6832-23_6832-3del (p.Gly2278_Gln2300del)	IVS86	87	dominant	c. =	NA	NA	NA	heterogeneous exon 87 skip	DDEB-ac	normal[d]	this paper (EB-156)
12	c.6846G>C (p.Gly2278_Gln2300del)	87	87	dominant	c. =	NA	NA	NA	heterogeneous exon 87 skip	DDEB-pr	normal[d]	17
13	c.6855_6881del (p.Gly2278_Gln2300del)	87	87	dominant	c. =	NA	NA	NA	heterogeneous exon 87 skip	DDEB-pt	normal[d]	18
14	c.6863_6878del (p.Gly2278_Gln2300del)	87	87	dominant	c. =	NA	NA	NA	heterogeneous exon 87 skip	DDEB-pr – DDEB-u	normal	19,20
				dominant	c.2005C>T (p.(Arg669*))	15	NA	recessive	homogeneous exon 87 skip	RDEB-gen sev	strongly reduced	50
				dominant	c.425A>G (p.(Thr90Serfs*4))	3	3	recessive	homogeneous exon 87 skip	RDEB-gen sev	unknown	20
15	c.6899A>G (p.Gly2278_Gln2300del)	87	87	dominant	c. =	NA	NA	NA	heterogeneous exon 87 skip	DDEB-pr	normal	21,22,51

(*Continued on next page*)

Table 1. *Continued*

No.	Allele 1[a]	Exon/ Intron	Skipped Exon	Dominant/ Recessive	Allele 2[a]	Exon/ Intron	Skipped Exon	Dominant/ Recessive	Functional Effect on COLVII[b]	Associated Phenotype	IF	References
16	c.6899_6900del (p.Gly2278_Gln2300del)	87	87	dominant	c. =	NA	NA	NA	heterogeneous exon 87 skip	DDEB-gen	normal[d]	this paper (EB-152)
17	c.6900G>A (p.Gly2278_Gln2300del)	87	87	dominant	c. =	NA	NA	NA	heterogeneous exon 87 skip	DDEB-ac	slightly reduced	23,24
18	c.6900+1G>C (p.Gly2278_Gln2300del)	IVS87	87	dominant	c. =	NA	NA	NA	heterogeneous exon 87 skip	DDEB-pr	normal	25
19	c.6900+1G>T (p.Gly2278_Gln2300del)	IVS87	87	dominant	c. =	NA	NA	NA	heterogeneous exon 87 skip	DDEB-pr – DDEB-u	unknown	26,27
20	c.6900+4A>G (p.Gly2278_Gln2300del)	IVS87	87	dominant	c. =	NA	NA	NA	heterogeneous exon 87 skip	DDEB-pr	unknown/ strongly reduced	27–30
				dominant	c.2044C>T (p.(Arg682*))	15	NA	recessive	homogeneous exon 87 skip	RDEB-gen sev	unknown	30
21	c.7929+1G>A (p.Gly2626_Lys2643del)	IVS106	106	recessive	c.7929+1G>A (p.Gly2626_Lys2643del)	IVS106	106	recessive	homogeneous exon 106 skip	RDEB-gen sev	strongly reduced	51
22	c.7930-1G>C (p.Gly2644_Met2661del)	IVS106	107	recessive	c.6527dup (p.(Gly2177Trpfs*113))	NA	NA	recessive	homogeneous exon 107 skip	RDEB-gen sev	undetectable	52
23	c.7894-2A>G (p.Gly2662_Lys2682del)	IVS107	108	dominant	c. =	NA	NA	NA	heterogeneous exon 108 skip	DDEB-gen	normal[d]	this paper (EB-339)
24	c.8045A>G (p.Gly2662_Lys2682del)	108	108	dominant	c. =	NA	NA	NA	heterogeneous exon 108 skip	DDEB-pt	normal	31
25	c.8227-1G>C (p.Gly2743_Gln2768del)	IVS110	111	recessive	c.1573C>T (p.(Arg525*))	12	NA	recessive	homogeneous exon 111 skip	RDEB-gen intermed	reduced[d]	this paper (EB-363)
26	c.8304+1G>A (p.Gly2743_Gln2768del)	IVS111	111	recessive	c.8717del (p.(Pro2906Leufs*46))	117	NA	recessive	homogeneous exon 111 skip	RDEB-ac	reduced	32
27	c.8524_8527+10del (p.Arg2814_ Glu2843delinsGln)	115	115	recessive	c.6127G>A (p.Gly2043Arg)	73	NA	dominant	exon 115 skip, mutant	RDEB-gen intermed	normal	53
				recessive	c.6025G>A (p.Gly2009Arg)	73	NA	unknown	exon 115 skip, mutant	RDEB-gen intermed	slightly reduced	53
				recessive	NF	NA	NA	NA	heterogeneous exon 115 skip	RDEB-pt	normal/ NC-2 retention[d]	54,55

DDEB-ac, DDEB-acral; DDEB-gen, DDEB-generalized; DDEB-pr, DDEB-pruriginosa; DDEB-pt, DDEB-pretibial; DDEB-u, DDEB-unknown (mild form); NA, not applicable; NF, not found; RDEB-ac, RDEB-acral; RDEB-gen intermed, RDEB-generalized intermediate; RDEB-gen sev, RDEB-generalized severe; RDEB-pr, RDEB-pruriginosa; RDEB-pt, RDEB-pretibial.

[a]Some of the DNA variants have been shown to lead to multiple splice variants at the RNA level. For reasons of readability, only splice variants deduced to lead to protein expression are shown between brackets. For instance, p.[=, Gly1925_Pro1940del]) indicates that the DNA variant c.5820G>A in exon 70 leads to two functional protein molecules, as deduced from RNA analysis: a wild-type protein isoform (p. =) and an isoform from which exon 70 is skipped (p.Gly1925_Pro1940del). More information on other non-functional splice variants can be found in the references cited. "c. =" means wild-type allele.

[b]Heterogeneous exon skip indicates there will be a combination of wild-type and skipped product. Mutant products (caused by a missense mutation) are mentioned explicitly, where present.

[c]Only the effect on the protein level and phenotype is provided, because no written consent was obtained.

[d]Retention of protein in basal keratinocytes was observed.

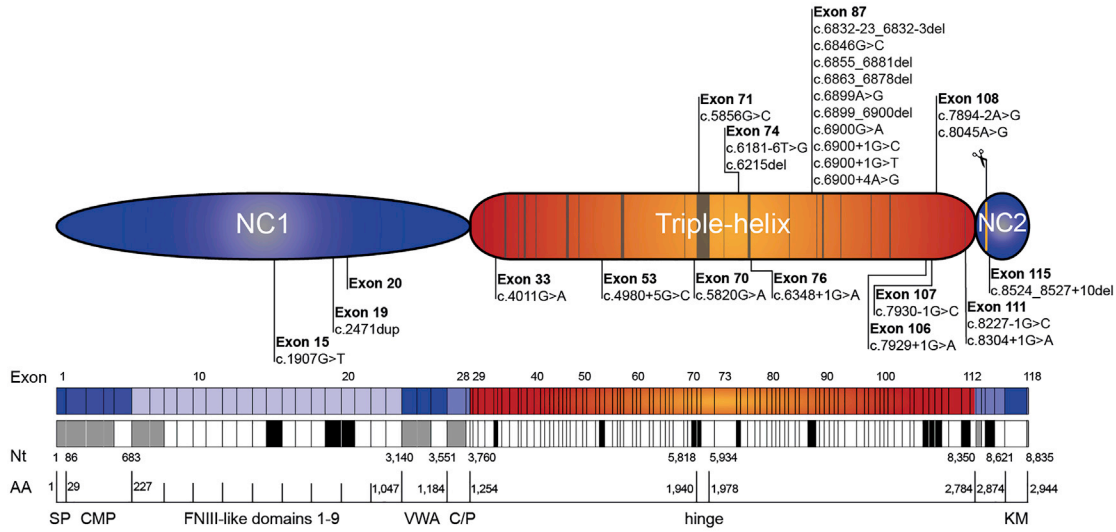

Figure 2. Overview of the *COL7A1* Gene and Natural Exon Skipping Variants

The upper pane shows an overview of the type VII collagen protein with the noncollagenous-1 (NC1), triple-helix (THD), and noncollagenous-2 domains (NC2). Dominant and recessive variants leading to natural exon skipping are indicated above and below the pane, respectively. Interruptions of the Gly-X-Y structure are indicated in gray to scale. The NC-2 cleavage site is indicated (scissors). In the middle pane, the upper bar shows all 118 exons to scale with the corresponding nucleotide (Nt) and amino acid (AA) numbers; colors correspond to the respective protein domains encoded. The second bar shows the same exon structure of *COL7A1* to scale, this time indicating the exons involved in natural exon skipping (black), other in-frame exon skipping candidates (white), and "non-skippable," out-of-frame exons (gray). The lower pane shows the relative location of crucial domains. CMP, cartilage-matrix protein motif; C/P cysteine/proline-rich motif; FN-III, fibronectin-III-like domains 1–9; hinge, intrinsically disordered hinge region; KM, Kunitz-motif-like domain; SP, signal peptide; VWA, von Willebrand factor A-like domain.

sites. A higher number of variants was found in splice donor sites than acceptor sites: 14 versus 9, respectively. The higher prevalence of donor site variants than acceptor site variants has been reported in a large cohort of splice-site variants.[33] There appeared to be no difference in mechanism between dominant and recessive variants. The other 4/27 variants exerted their exon skipping effect through disrupting predicted exonic splicing enhancer sequences (ESEs): two small deletions and one single base substitution located in an ESE in the center of exon 87.[17–19] ESEs are the class of splicing signals that AONs aim to target in order to induce exon skipping.

Genotype-Phenotype Correlation

Dominant Exon Skipping

In total, 15 exon skipping variants were found to be associated with dominant phenotypes (17 patients). The overall clinical heterogeneity observed in dominant exon skipping cases was similar to that observed for dominant glycine substitutions.[12] The dominant phenotypes that were observed with skipping of exons 71, 74, 87, and 108 almost cover the complete DDEB phenotypic spectrum from acral, pretibial, pruriginosa, to DDEB-gen. The phenotypic spectrum of DDEB phenotypes caused by exon skipping could not be explained by differences in the amount of detectable type VII collagen at the BMZ, because this was reported to be normal for 13 out of 15 variants. The only two variants for which type VII collagen expression was reported to be abnormal led to slightly reduced and strongly reduced expression, and the acral and pruriginosa forms of DDEB, fitting in the middle of the DDEB-phenotypic spectrum, respectively. Dominant skipping of exons 71, 74, and 108 resulted in the pretibial, pruriginosa, and generalized forms of DDEB, respectively. Skipping of exon 87 was associated with a variety of phenotypes, leading to DDEB-ac, DDEB-pretibial (DDEB-pt), DDEB-pruriginosa (DDEB-pr), and DDEB-gen (Table 1). To some extent, dominant skipping leads to the retention of type VII collagen in basal keratinocytes, as shown by IF (Figure 1).

In contrast with recessive exon skipping variants that were found throughout the gene, and in concert with classical DDEB-causing glycine substitutions, the 15 dominant exon skipping variants were located exclusively within the THD, predominantly in its C-terminal region. More specifically, they were all located in, or in the vicinity of, interruptions in the collagenous structure: (1) either in or directly adjacent to the hinge region (exons 71 and 74, respectively), or in close proximity to (2) interruptions in the THD or (3) the NC-2 domain (exons 87 and 108, respectively). Exon 87 (Figure 3) represents a hotspot for dominant natural exon skipping variants: 10 different variants having been identified to date that lead to skipping of exon 87. Exon 87 precedes a short imperfection in the collagenous structure, located 2 amino acids downstream in exon 88, and a larger imperfection of 18 amino acids farther downstream in exon 89.

Recessive Exon Skipping

Recessive exon skipping variants were scattered throughout the gene, and there was no apparent distribution pattern related to interruptions of the glycine repeat of the THD (Figure 2). Exons 70 and 73

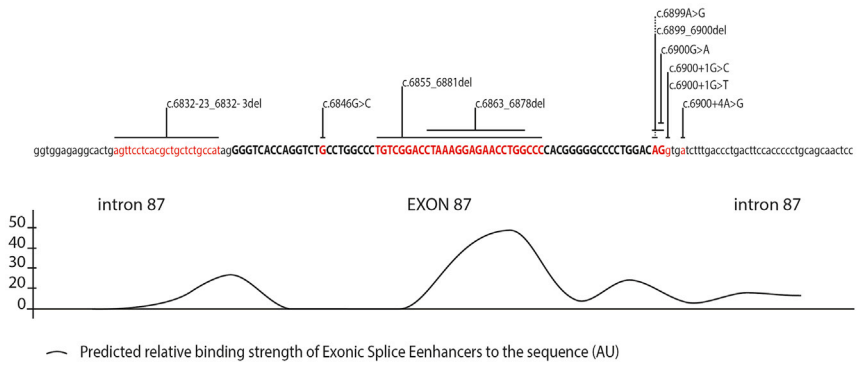

Figure 3. Variants Causing Natural Exon 87 Skipping
Exon 87 and its flanking intronic sequences showing variants that cause natural exon skipping in red. Predicted binding strength of exonic splice enhancers is shown in the lower graph. AU, arbitrary units.

are located immediately upstream and downstream of the hinge region (encoded by exons 71 and 72). Exon 111 contains a minor interruption and is located only 15 amino acids upstream of the NC-2 domain, whereas exons 33, 53, 95, 106, and 107 are separated by 18, 48, 15, 108, and 90 amino acids from interruptions in the glycine repeat, respectively.

In total, 12 exon skipping variants (20 patients) were found to be associated with recessive phenotypes. All RDEB phenotypes were observed: i.e., acral, pretibial, generalized intermediate, and even the generalized severe form of RDEB. Fourteen patients had phenotypes milder than the most severe phenotype (2 patients had acral, 1 pruriginosa, 1 pretibial, and 10 gen intermed RDEB), whereas six patients were reported as having RDEB-generalized severe. In contrast with dominant exon skipping variants where, in general, normal expression levels of type VII collagen were observed, recessive variants resulted in varying expression levels ranging from complete absence to normal (Table 1). As expected, type VII collagen expression correlated well with phenotypic severity (Figure S2). Normal type VII collagen expression was seen in patients with acral, pretibial, and generalized intermediate forms, whereas it was "slightly reduced" or "reduced" in patients with generalized intermediate forms of RDEB. Strongly reduced expression was observed in generalized intermediate and generalized severe forms of RDEB. Complete absence of type VII collagen expression was reported in only one case with generalized severe RDEB. Complete absence of type VII collagen expression levels was an unexpected finding given that the exon skipping mutation was expected to preserve protein production. This could be explained by low-grade exon 107 skipping, resulting in undetectable levels of type VII collagen, or an uncovered null mutation *in cis* with the exon 107 skipping variant.

Of particular interest, with regard to exon skipping therapy, is variant 1 (Table 1). The respective patient carried the variant c.2005C>T (p.Arg669*) in exon 15 and a frameshift variant c.6311_6312del (p.Ser2104Trpfs*12) in exon 76. This genotype was predicted to go with complete absence of type VII collagen and, therefore, the most severe RDEB-gen sev phenotype. However, the patient also carried the rare single-nucleotide variant c.1907G>T (rs116005007, minor allele frequency 0.22%: T = 0.0022/11; 1000 Genomes; https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/) *in cis* with the c.2005C>T variant. This variant, located in the first codon of exon 15, was subsequently shown to modify splicing of exon 15, leading to in-frame skipping of exon 15, thus bypassing the null variant and resulting in the synthesis of some functional type VII collagen, which explained the substantially milder phenotype. The carrier parent had no visible DEB features.

In contrast with 14 patients with milder phenotypes, 6 out of 20 cases from the literature were described as having a severe generalized RDEB phenotype (Table 1). In all six cases, type VII collagen expression was either strongly reduced or completely absent. Five of the six cases were compound heterozygous for a null variant *in trans* with the exon skipping variant. In contrast, of the 14 patients with milder phenotypes, only 6 carried a null allele *in trans* with the exon skipping variant, and 8 had two functional alleles (either a homozygous exon skipping variant or a combination of the exon skipping variant with a missense variant). Only one patient with RDEB-gen sev carried the exon skipping variant homozygously. This homozygous variant, c.7929+1G>A, was associated with strongly reduced expression of an exon 106-skipped type VII collagen. Unfortunately, detailed individual data were not available to assess and compare the expression levels and the phenotypes of these cases.

DISCUSSION

We investigated the genotype-phenotype correlation of natural exon skipping variants found in patients with DEB to anticipate the therapeutic effect of AON-mediated exon skipping. We included a series of seven *COL7A1* exon skipping variants from the Dutch EB registry and 20 additional exon skipping variants from the literature. For all 27 variants, exon skipping had been confirmed at the RNA level.

Our review shows that natural exon skipping variants act either autosomal dominantly or recessively (Figure 2). Altogether, the phenotypic spectrum caused by dominant exon skipping reflects the normal phenotypic spectrum of DDEB. In contrast, recessive exon skipping generally leads to a phenotype on the milder end of the RDEB-phenotypic spectrum. However, the expression levels of the skipped proteins appear to be very important for the precise phenotypic outcome. Assessing the exact correlation between expression of the skipped type VII collagen and the clinical phenotypes was, however, not straightforward, because there was considerable variation in reported protein expression levels as determined by IF. This is at least partly due to the fact that patients were analyzed in different clinics, by different clinicians, and at different

ages. Although systematic analysis at the RNA and protein levels for all natural exon skipping mutations was not possible, the general pattern that emerged was that the expression levels of type VII collagen lacking an exon in either the NC-1 domain or the THD correlated well to the severity of the phenotype, comparable with other recessive mutations. In addition, individual cases show that low levels of expression of type VII collagen can already dramatically ameliorate the phenotype.

Our study therefore sets the stage for AON-mediated exon skipping, because these results indicate that exon skipping will most likely not have a beneficial effect on DDEB caused by glycine substitutions. Unless mutation-specific, AON-mediated exon skipping will most likely skip both mutant and wild-type exons and, if this process were 100% efficient, would change a heterozygous, dominant-negative glycine substitution into a homozygous exon skipping mutation. Although this would not have the problem of dominant-negative interference, homogenously expressing exon-skipped type VII collagen is not expected to improve the DDEB phenotype. This is illustrated by 10 patients who homogeneously expressed type VII collagen lacking exons 15, 19, 20, 33, 53, 87, 106, 107, or 111 (Table 1) and whose phenotypes were more severe than DDEB phenotypes at the severe end of the spectrum. Although this could partly be due to lower-than-normal type VII collagen expression and may not be true for all exons, the natural exon skipping data so far do not provide evidence that exon skipping would benefit DDEB phenotypes. Furthermore, because AON-mediated exon skipping will be significantly less efficient than 100% and cannot be administered continuously, treatment of DDEB with exon skipping will likely introduce an exon-skipped allele in addition to the wild-type and mutant alleles, which might even worsen the phenotype. Therefore, other therapeutic strategies seem to be more appropriate for DDEB, such as allele-specific knockdown using siRNAs.[34]

In contrast, although exon skipping might not work for all exons, as illustrated by the cases with a severe generalized phenotype, AON-mediated exon skipping could be beneficial for RDEB-gen sev patients, where complete absence of type VII collagen is the cause of disease. Two cases well illustrate what exon skipping as a therapeutic approach aims to achieve, i.e., bypassing a null variant by excluding its residential exon. The null variant c.2005C>T in exon 15 was rescued by the rare DNA variant c.1907G>T in the same exon, which causes skipping of exon 15 and thus bypasses the null variant.[35] Although the level of expression of type VII collagen lacking exon 15 was still rather low, the co-occurrence of both variants on the same allele led to a milder than expected RDEB-gen intermed phenotype. The second case had the c.2471dup variant in exon 19, which was predicted to cause a frameshift with premature termination codon and the most severe phenotype. However, skipping of exon 19 led to moderate levels of exon 19-skipped type VII collagen, and a surprisingly mild RDEB-gen intermed phenotype.[36] These exon 15 and exon 19 skipping cases show that the introduction of even small amounts of skipped type VII collagen can result in significantly less severe phenotypes.

Why some natural exon skipping variants act dominantly and others recessively is unknown. In general, and comparable with dominant glycine substitutions,[37] dominant skipping exons are located closer to collagenous imperfections than recessive ones and are found more toward the end of the THD. However, location does not seem to be the only reason, because two of the recessive exons are also located near collagenous imperfections, and five are located near the end of the THD. A possible explanation as to why some skipped exons act dominantly whereas others act recessively could be the ratio between exon-skipped alleles and wild-type alleles. Analogous to the finding that the wild-type versus mutant allele ratio determines the level of THD instability and consequently the phenotype,[38] it is conceivable that the relative amount of exon-skipped type VII collagen present is also crucial in determining its phenotypic effect. Unfortunately, it is not possible to study this hypothesis in detail because the level of exon skipping was quantified at the RNA level for only one of the exon skipping variants (variant 9 in Table 1). The heterozygous variant c.6215delA resulted in a 0.73:1 exon 74-skipped to wild-type type VII collagen mRNA ratio and the DDEB-pr phenotype.

Intra-epidermal cytoplasmic retention of type VII collagen has been described for several variants and is believed to be the result of disturbed triple-helix formation. For instance, it has been reported for several glycine substitutions leading to DDEB, like the p.Gly2037-Glu glycine substitution,[39] bullous dermolysis of the newborn,[40] and RDEB-inversa.[41] The fact that we observed cytoplasmic retention in all four dominant exon skipping cases and in one of the three recessive skipping cases indicates that exon skipping also disturbs normal triple-helix formation because of a dominant-negative effect. Hence it makes sense that the dominant phenotypes due to natural exon skipping fall in the range seen with glycine substitutions. Intra-epidermal retention in a skin biopsy of a DEB patient with unknown genetic cause could thus also point to in-frame exon skipping and should warrant RNA analysis if no glycine substitution is found.

Clearly, exon 87 is a hotspot for dominant natural exon skipping. Ten different variants that induce skipping of exon 87 were identified, of which 6 are located in the intron 87 splice donor site, indicating that this is a weakly defined exon. It has been shown that variants in donor sites are more likely to disrupt splicing, and that the lack of cryptic splice donor signals within 50 bp downstream of splice donor sites increases the likelihood of skipping the entire exon.[33] Using Human Splicing Finder[42] (http://www.umd.be/HSF3/), we examined exons 86, 87, and 88 (Figure S3) for such cryptic splice donor sites. Indeed, no cryptic splice donor sites were predicted downstream of the splice donor site of exon 87, whereas three and seven sites were found for exons 86 and 88, respectively. The lack of these cryptic splice donor sites in intron 87 may explain why so many variants lead to complete skipping of exon 87.

Pruritus is strongly linked to disorders of the BMZ, including EB. An association has been suggested between heterozygous skipping of exon 87 and pruritus.[23] Indeed, six patients with exon 87 skipping

variants were reported to have the DDEB-pr phenotype. One other case of DDEB-pr was found to have an exon skipping mutation, exon 71. Four of the seven families originated from Southeast Asia,[16,22,25,26] a geographic region where pruritic diseases are more common in general.[43] Whether there exists a causative link between exon skipping and DDEB-pr, and what the underlying mechanism would be, or that this association is merely due to a combination of other genetic and environmental factors, cannot be answered from this review and needs further study.

RDEB-gen intermed is still a severe phenotype in itself and improving a RDEB-gen sev phenotype to this level is still far from curing RDEB. However, we believe that preventing the development of the RDEB-gen sev phenotype would be a major improvement for several reasons: reduced cancer risk, a longer lifespan, a reduced risk and slower progression of pseudosyndactyly and esophageal strictures, and thus improved quality of life.

In conclusion, exon skipping therapy for DDEB patients seems unlikely to benefit patients and may theoretically even worsen their phenotype, whereas such therapy for RDEB patients has the potential to improve the RDEB-gen sev phenotypes, particularly caused by bi-allelic null mutations, and push the clinical outcome toward the milder RDEB-gen intermed phenotype. The focus of developing exon skipping as a therapeutic approach should therefore be on RDEB-gen sev patients because of *COL7A1* null variants.

MATERIALS AND METHODS

Molecular Analysis of Patient Material

Molecular analysis at the DNA and protein level was performed as previously described.[2] In short, DNA, isolated from peripheral blood lymphocytes, was subjected to variant analysis of the *COL7A1* gene (GenBank: NM_000094.3) by direct Sanger sequencing. Protein expression was examined by IF microscopy on 4-μm cryosections using monoclonal LH7.2 antibody (Abcam, Cambridge, UK) and analyzed on a Leica DMRA microscope (Wetzlar, Germany). Randomly primed two-step RT-PCR was performed on RNA isolated from 40-μm skin cryosections, using PCR primers that bind to exons at least two exons upstream or downstream of the variant, in order to be able to identify potential skipping of multiple exons.

Dutch EB Registry and Literature Review

To gain a complete picture of *COL7A1* variants that cause in-frame exon skipping, we scrutinized the Dutch EB registry, the DEB registry (https://www.deb-central.org), and the literature on DEB and *COL7A1*. "COL7A1," "COL7A1 splicing," and "COL7A1 exon skipping" were used as queries in a search of NCBI PubMed (https://www.ncbi.nlm.nih.gov/pubmed). All variants that led to skipping of complete exons were included, regardless of the presence of additional splice variations. We excluded variants that led only to splice patterns other than in-frame exon skipping (i.e., skipping of an out-of-frame exon, in-frame or out-of-frame deletions or insertions not involving an entire exon, or partial or full intron retention). Confirmation of exon skipping at the RNA level was a prerequisite for inclusion. Reported expression levels were unified into normal/slightly reduced/reduced/strongly reduced/absent.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.09.009.

AUTHOR CONTRIBUTIONS

J.B. performed RNA and protein analysis, literature research, and prepared the manuscript. E.H.v.d.H. performed literature research. D.S.E. performed RNA and protein analysis. R.M., H.H.L., H.S., and R.J.S. performed DNA sequencing and downstream analysis. M.F.J. provided clinical supervision. A.M.G.P. provided concept and manuscript supervision. P.C.v.d.A. provided concept, clinical, project, and manuscript supervision.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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